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Negative Regulatory Element in the Mammary Specific Whey Acidic Protein Promoter

Andreas F. Kolb, Walter H. Günzburg, Richard Albang, Gottfried Brem, Volker Erfle, and Brian Salmons

Lehrstuhl für Molekulare Tierzucht, Ludwig-Maximilians Universität, D-80539 München, Germany (A.F.K., G.B., B.S.) and Institut für Molekulare Virologie, GSF-Forschungszentrum für Umwelt und Gesundheit, D-85758 Oberschleissheim, Germany (W.H.G., R.A., V.E.)

Abstract Expression of the whey acidic protein (WAP) gene is tightly regulated in a tissue and developmental stage specific manner, in that the WAP gene is exclusively expressed in the mammary gland during pregnancy and lactation. Using both deletion and competition analyses, evidence is provided for the existence of a negative regulatory element (NRE) in the WAP promoter located between −413 and −93 with respect to the WAP transcriptional initiation site. This NRE dramatically decreases transcription from linked heterologous promoter-reporter gene constructs. The activity of NRE requires WAP promoter sequences that are 230 bp apart since subfragments of the NRE fail to inhibit transcription of adjoining reporter genes. Nuclear extracts from different cell types, in which the WAP gene is not active, contain a protein or complex that specifically interacts with the entire NRE but not with subfragments of it. The contact points between this protein (NRE binding factor [NBF]) and the NRE element have been partially determined. Mutation of the implicated nucleotides severely reduces the ability of NBF to bind, and such mutated promoter fragments fail to alleviate transcriptional repression in competition experiments. This suggests that NBF binding to the NRE is at least in part responsible for the negative regulation of the WAP promoter. Since NBF is not detectable in the lactating mammary gland, where the WAP gene is expressed, we speculate that it may be a determinant of the expression spectrum of the WAP gene.

Key words: negative regulatory element, heterologous promoter, DNA binding factor, transcriptional repression, milk protein expression control

Rodent milk contains an abundant protein, whey acidic protein (WAP), which is present in the whey fraction [Hennighausen and Sippel, 1982]. The expression of WAP is confined to the mammary glands of pregnant and lactating but not virgin mice [Piletz et al., 1981; Dandekar et al., 1982; Hennighausen et al., 1982; Hobbs et al., 1982]. At least some of the regulatory domains that confer this tissue specific and developmentally regulated expression reside within the 5′ proximal sequences of the WAP gene, since a 2.4 kb WAP promoter fragment effectively directs mammary gland specific expression of a variety of linked heterologous genes during pregnancy and lactation in transgenic mice [reviewed by Hennighausen, 1990]. Generally, however, the expression levels observed from such transgenes are lower than that from the endogenous WAP gene, suggesting an involvement of additional regulatory elements located elsewhere. This notion is supported by recent studies in which transgenes carrying promoter and intragenic and 3′ untranslated sequences of the WAP gene have given site of integration independent, high level expression in transgenic mice [Bayna and Rosen, 1990; Burdon et al., 1991; Dale et al., 1992].

Pregnancy hormones are implicated in the regulation of WAP expression, and this has been confirmed using mammary gland derived organ culture systems [Pittius et al., 1988]. In this system, the concerted action of the lactogenic hormones, insulin, hydrocortisone, and prolactin are required for high level expression from the WAP promoter [Pittius et al., 1988]. More recently, two hormone response elements have been identified in the mouse WAP promoter as being located in two regions, from the transcrip-

Andreas F. Kolb's present address is Institut für Virologie und Immunologie, Universität Würzburg, Versbacherstraße 7, D-97078 Würzburg, Germany.

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Address reprint requests to Brian Salmons, Institut für Molekulare Virologie, GSF-Forschungszentrum für Umwelt und Gesundheit, D-85758 Oberschleissheim, Germany.

tion initiation site to −450 and from −550 to −1,100, respectively [Doppler et al., 1991], though this contrasts with results obtained from the rabbit WAP which implicated a region between −1,806 and −3,000 as containing hormone response elements [Devinoy et al., 1991]. However, lactogenic hormones alone are presumably not sufficient for the induction of WAP expression in the mammary gland since many other organs and cell types respond to pregnancy hormones but yet are not able to express the WAP gene [Topper and Freeman, 1980]. Thus additional regulatory mechanisms must play a role in determining the tissue specific expression spectrum of this gene. Indeed, evidence has been provided for nonhormonal factors that regulate transcription, and enhancer responsive elements have been identified that may be involved in the regulation of WAP promoter activity using in vitro transcription assays [Lubon and Hennighausen, 1987; Lubon et al., 1989].

In addition to positively acting transcription factors, negative regulation or repression of transcription has been shown to play a role in determining temporal developmental and tissue specific regulation of the expression of an ever increasing number of genes [for a review see Clark and Docherty, 1993]. Mechanisms that negatively regulate transcription prevent inappropriate expression of gene products. A gene such as WAP may be expected to be subjected to such regulatory mechanisms to ensure that it is not expressed in hormone responsive tissues or organs other than the mammary gland. The aim of the studies reported here was to define and characterize such regulatory mechanisms that contribute to the control of WAP expression.

One of the major problems that has hampered the elucidation of regulatory mechanisms involved in controlling the expression of WAP (and certain other milk protein genes) has been the difficulty in finding cell culture systems that can express either the endogenous WAP gene or reporter gene constructs linked to the WAP promoter [Chen and Bissell, 1989; Doppler et al., 1991; Dale et al., 1992]. The inability of cell culture systems to mimic the normal architecture and cell-cell interactions found in the mammary gland is thought to be one reason for the inability of these cells to express certain milk protein genes, including WAP [Eisenstein and Rosen, 1988; Reichmann et al., 1989; Chen and Bissell, 1989]. This phenomenon may be due to the repression of expression from the WAP promoter in such cells.

Here we show that the WAP promoter contains a negative regulatory element (NRE) that represses expression from the reporter gene promoter. Further, the NRE is shown to interact with a nuclear protein (NRE binding factor [NBF]) that is abundant in all cell lines and tissues examined in which the WAP gene is not expressed but only poorly or not at all expressed in the lactating mammary gland where the WAP gene is expressed. The interaction between NBF and the regulatory element located in the WAP promoter are candidate determinants of the tissue specificity of WAP expression.

METHODS

Plasmid Constructs

The plasmids WAP1-hGH and WAP2-hGH (Fig. 1A) have been previously described [Günzburg et al., 1991]. The luciferase expression plasmids (pT.Eco/Sac, pT.Sac/Xho, pT.Xho/Kpn, pT.Xho/Xba, pT.Xho/EcoN, and pT.EcoN/Xba) were generated by ligating the indicated WAP promoter subfragments into the multiple cloning site of the plasmid pT109luc (Fig. 2A) kindly donated by Steven Nordeen [Nordeen, 1988]. This plasmid carries a firefly luciferase gene under the transcriptional control of the herpes simplex virus thymidine kinase (tk) gene promoter and was designed for the testing of the ability of DNA fragments to influence transcription. Some of the WAP promoter fragments (Xho/Kpn, Xho/Xba, Xho/EcoN, and EcoN/Xba) were inserted in both the same and opposite orientation with respect to the tk promoter. The plasmids used for the competition assay (pUC.Eco/Sac, pUC.Sac/Sac, pUC.Xho/Xba, pUC.Sac/Xba, and pUC.Xho/Sac) were generated by digestion of pWAP2-hGH with the indicated restriction enzymes and ligation of the respective WAP promoter fragments into pUC19 previously digested with the relevant enzymes. Poly3-WAP, containing the 2.4kb WAP promoter cloned into the EcoRI/KpnI sites of pUC19, was kindly provided by Mathias Müller (University of Munich).

PCR Mediated Site Directed Mutagenesis

Mutated WAP promoter fragments were generated by PCR amplification of a WAP promoter

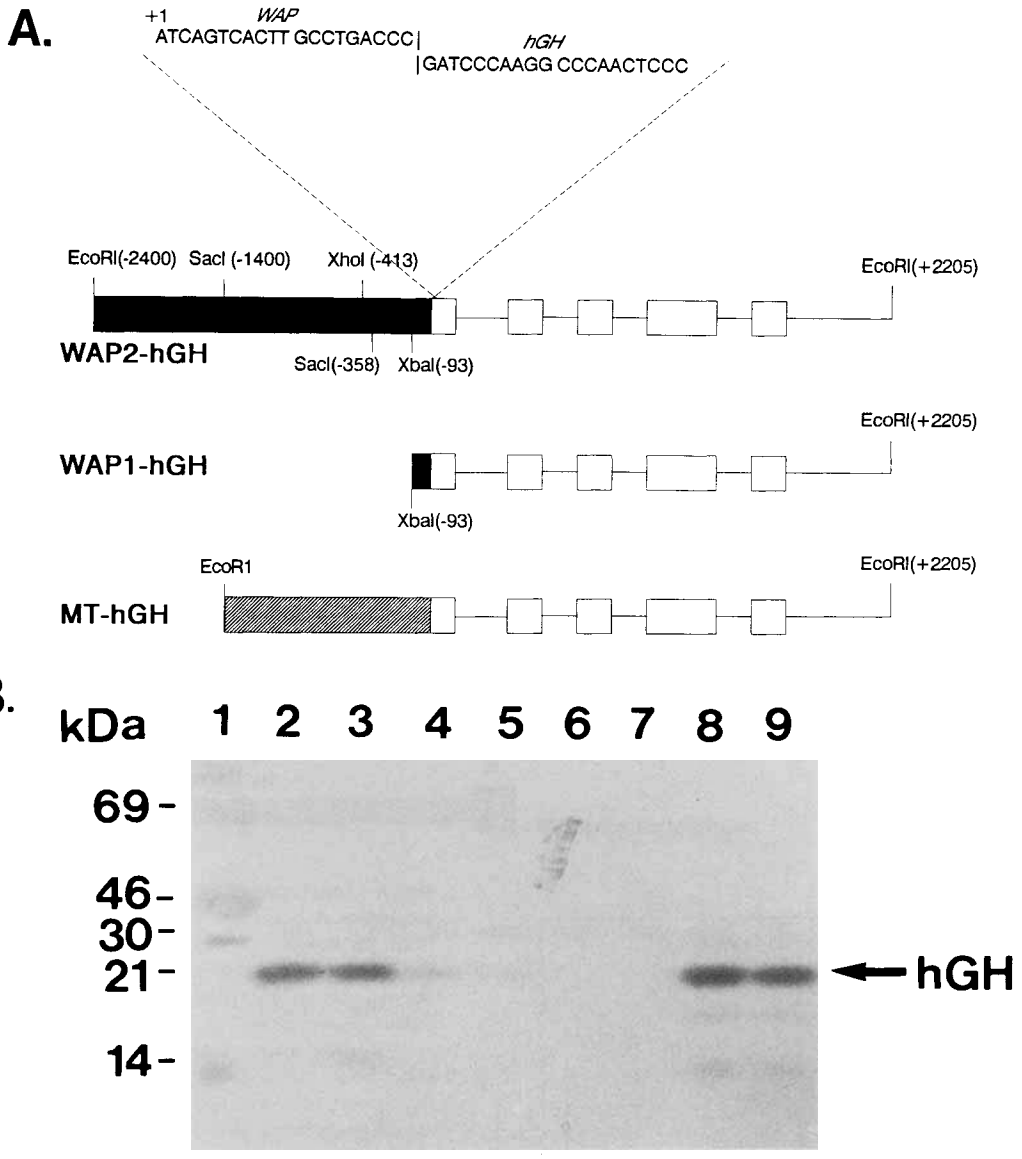
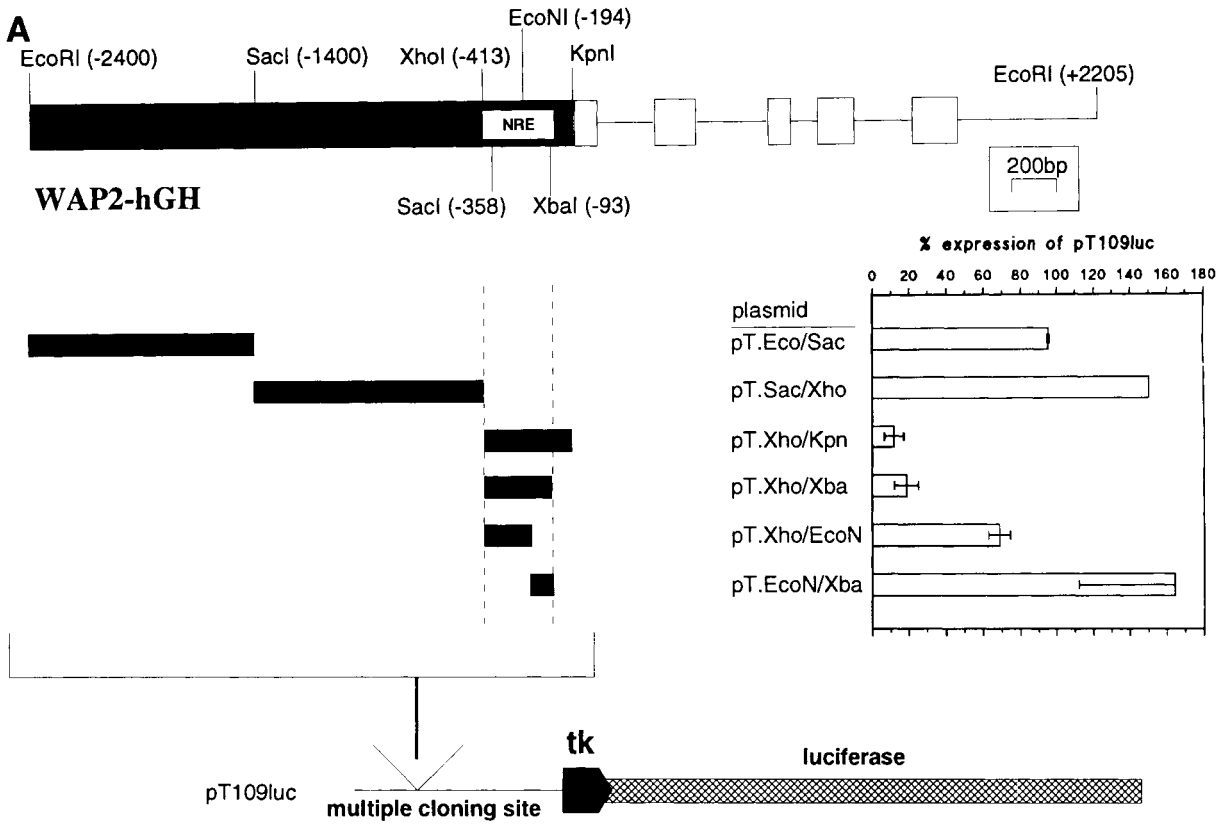


Fig. 1. Structure and expression of WAP-hGH reporter gene constructs. **A:** Schematic representation of the plasmids WAP2-hGH (containing 2.4 kb of WAP 5′ flanking sequences linked to the human growth hormone coding region), WAP1-hGH (containing 92 bp of WAP promoter sequences), and MT-hGH (carrying the human growth hormone under the transcriptional control of the metallothionein promoter). WAP promoter sequences are represented as black boxes, the metallothionein promoter sequence as a striped box, and the exons of the hGH coding sequences as open boxes. Important restriction sites are indicated relative to the first nucleotide of the hGH coding region. The sequence at the junction between the WAP and hGH fragments was determined and is shown. Originally, the WAP sequence terminated in a KpnI site. The 3′ overhang was removed by exonuclease digestion during which the first nucleotide of the KpnI site (G) was also removed. The hGH sequence, which terminated in a BamHI site, was cleaved with BamHI and the 5′ overhang filled in using Klenow. The WAP and hGH fragments were then blunt end ligated. **B:** Western blot analysis of hGH protein expression after transfection of WAP1-hGH (**lanes 2,3**), WAP2-hGH (**lanes 4,5**), pSV2neo (**lanes 6,7**), or MT-hGH (**lanes 8,9**) into CK cells. Membrane protein extracts were harvested after transfection and subjected to Western blot analysis. The position of the 22 kD hGH protein is indicated, as are the positions of marker proteins shown in lane 1.

fragment from −415 to −163 using primers complementary to the WAP promoter sequence as well as two primers carrying the following underlined base changes: Primer 1, 5′-CGGGCGCCAGGAACAAGGGCCACAATGGAATT CATTTTTA-3′ (carrying a conversion from GGC to ATT at positions −167 to −169); Primer 2, 5′-AAGTCGACGTTTGCTCAAACCTCCTGTCTTGTTTTCTAT-3′ (carrying a C to G conversion at position −413 and a GCC conversion to a CGT at positions −408 to −406, relative to the published transcriptional start site of hGH).



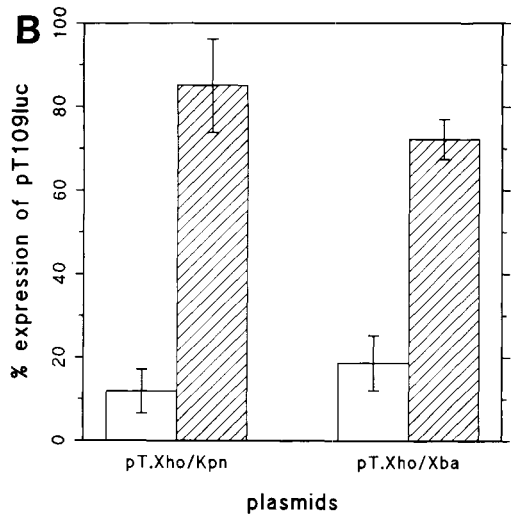


Fig. 2. Repression of TK promoter activity by WAP promoter sequences. **A:** Schematic representation of the plasmids WAP2-hGH, the reporter plasmid pT109luc, and the WAP promoter fragments inserted into pT109luc. WAP promoter sequences are represented by black boxes and exons 1–5 of the hGH coding region in the WAP2-hGH plasmid by open boxes. The herpes simplex virus thymidine kinase promoter (tk) is indicated by a black arrow and the luciferase coding sequences (luciferase) by a hatched box. The putative location of the negative regulatory element (NRE) is indicated. Luciferase expression after transfection of CK cells was normalized to the expression of the MT-hGH internal control plasmid and is presented as the mean of the percentage of luciferase activity obtained with the plasmid pT109luc in three independent experiments. **B:** Effect of orientation on activity of the NRE. The WAP promoter fragments Xho/Kpn and Xho/Xba were inserted into pT109luc in the same (open boxes) or opposite (shaded boxes) orientation as the tk promoter. Luciferase expression after transient cotransfection of CK cells with the luciferase and MT-hGH plasmids was normalized to hGH expression and is presented as the mean of the percentage of luciferase activity obtained with pT109luc in three independent experiments.

Cell Culture and Transfection

Cells were routinely cultured in Dulbecco's modified Eagles medium containing 3.7% $NaHCO_3$ and 10% fetal bovine serum and incubated at 37°C (100% humidity and 5% CO_2). The medium was supplemented with 1 μg/ml insulin for cultivation of EF43 cells as previously described [Günzburg et al., 1988]. Transfection of the various plasmids was performed by calcium phosphate coprecipitation technique using a transfection kit as outlined by the supplier (Pharmacia, Freiburg, Germany). Transfections were performed with at least two independently prepared plasmid preparations for each construct analysed. Briefly, 5 μg circular plasmid DNA was used to transfect 5×10^5 cells that were cultivated in 6 cm petri dishes (Nunc, Wiesbaden, Germany). Sixteen hours posttrans-

fection cells were washed twice with PBS, and fresh medium was added. Luciferase activity and hGH protein levels were determined 48 h later. The expression data were normalized either by cotransfection of pT109luc and subsequent analysis of luciferase expression levels for hGH expressing test plasmids or by cotransfection of pMT-hGH [Günzburg et al., 1991] and subsequent analysis of hGH expression levels for luciferase expressing test plasmids. In both cases cotransfections were performed using a 1:3 ratio of control and test plasmid and the expression values of the test plasmid corrected relative to the expression of the control plasmid. For stable transfections, 0.5 μg of the plasmid pSV2neo [Southern and Berg, 1982] was added to the transfection mix. The transfection procedure was performed exactly as for transient transfection assays except 24 h after addition of fresh medium the cells were transferred to 226 cm^2 tissue culture flasks and 24 h later medium containing the antibiotic G418 (500 μg/ml) was added. After selection an average of 100 stable clones was pooled as a population and expanded.

Determination of hGH Protein and Luciferase Activity

The amount of hGH protein in cell culture medium was directly assayed using commercially available kit for ELISA (elias-Medizintechnik, Freiburg, Germany), according to the manufacturers' instructions. The integrity of the hGH protein was verified by Western blotting analysis as previously described [Janka et al., 1993] using a polyclonal rabbit anti-hGH serum (Paesel & Lorei, Frankfurt, Germany). Cells transfected with the luciferase constructs were scraped in a buffer containing 25 mM K_2HPO_4/KH_2PO_4 (pH 8.0), 8 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1% BSA, 1% Triton X-100, and 15% glycerol. Crude cell fragments were pelleted by short centrifugation at room temperature in a benchtop centrifuge and the supernatant assayed for luciferase activity [Nordeen, 1988]. Light emission at 562 nm was measured in a Luma-counter (Berthold, Wildbad, Germany) after addition of luciferin and Mg-ATP to final concentrations of 75 μM and 0.3 mM, respectively.

Gel Retardation Analysis of DNA-Protein Interactions

Crude nuclear extracts were prepared according to the method of Dignam [Dignam et al., 1983]. Total protein concentrations were determined by Bradford assay (Bio-Rad, München, Germany). Different amounts of these extracts were preincubated at room temperature with varying concentrations of nonspecific (Deisenhofen, Germany) competitor DNA (double stranded poly dldC; Sigma) in 10 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, and 2 mM DTT for 15 min. Approximately 1 ng (10,000 cpm) of a γ-^{32}P-ATP endlabeled DNA probe was added, and the samples were incubated at room temperature for a further 25 min to allow binding to occur and then separated on a native 6% polyacrylamide gel in a Tris-Borate-EDTA buffer system. The radioactively labeled DNA and DNA-protein complexes were visualized by autoradiography against X-OMAT (Kodak, Rochester, NY) film.

Nucleotide Sequencing

The mouse WAP promoter region was sequenced using a Sequenase II kit (USB, Bad Homburg, Germany). This sequence can be found in the GenBank data base under the accession number L21193.

Methylation Interference Assay

Methylation interference assays were performed essentially as described by Baldwin and Sharp [1988]. Briefly, 15 ng (or 1×10^6 cpm) of a γ-^{32}P-ATP labeled DNA fragment was methylated for 5 min by treatment with 1 μl DMS (100%) in 50 mM sodium cacodylate (pH 8.0), 1 mM EDTA (pH 8.0). The reaction was stopped by addition of 40 μl of 1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol. After addition of 10 μg of tRNA, the DNA was precipitated by addition of 2.5 volumes of 100% ice-cold ethanol. The sample was centrifuged immediately, washed twice with 70% ethanol, and dried in vacuo. The DNA was then used for gel shift binding reactions as described above. After resolution on a 6% nondenaturing polyacrylamide gel and exposure of X-ray film to the gel, the DNA and DNA-protein complexes were identified and extracted from gel slices as described [Sambrook et al., 1982]. The DNA isolated from the gel was then resuspended in 100 μl of 1 M piperidine and incubated at 95°C for 30 min and freeze dried. The dried pellets were resuspended in 100 μl of sterile water, freeze dried, and resuspended in a formamide sample buffer (80% deionized formamide, 50 mM Tris borate EDTA (pH 8.3) (1× TBE), 0.1% xylene cyanol, and 0.1% bromophenol blue). After denaturation, the reaction products were separated on a 6% denaturing polyacrylamide gel. Labeled DNA fragments were visualized by autoradiography.

RESULTS

WAP Promoter Contains a Negative Regulatory Element

Two chimeric constructs consisting of WAP 5′ sequences linked to the human growth hormone (hGH) reporter gene (Fig. 1A) were stably transfected into feline kidney (CK) [Crandell et al., 1973] cells and the culture medium assayed for hGH protein expression. As a control, CK cells were also transfected with a construct in which hGH coding sequences are linked to a strong, metallothionein promoter. The hGH coding region (Fig. 1A) carries a promoter within the first intron that directs hGH protein expression upon transfection into cultured cells in the absence of a linked heterologous promoter (Kolb et al., submitted) and thus can be used as a reporter for cis-acting regulatory elements.

Western blot analysis of medium from CK cells transfected with the MT-hGH construct reveals a secreted hGH protein of around 22 KDa (Fig. 1B, lanes 8,9). The amount of hGH synthesized by CK cells transfected with hGH coding sequences linked to the shorter WAP sequences (WAP1-hGH) (Fig. 1B, lanes 2,3) is similar to that from the MT-hGH construct and much greater than that obtained from the longer WAP containing construct (WAP2-hGH) (Fig. 1B, lanes 4,5). Quantitatiion of hGH production from these cells by ELISA revealed that the hGH expression from the WAP2-hGH construct (0.75 ng hGH/10^6 cells) is around 5% of that from the shorter WAP1-hGH construct (14.9 ng hGH/10^6 cells). Similar results were obtained upon transfection of the constructs into a number of different cell lines including NIH3T3 cells (not shown). These data suggest that a negative acting element is located between the Eco RI site (−2,400) and the Xba I site (−93) in the WAP promoter region (Fig. 1A). The differences in hGH protein expression are reflected at the RNA level, as demonstrated by both S1 analyses and runoff experiments (data not shown) confirming that the regulation is exerted at the transcriptional level.

Definition of the Negative Regulatory Element (NRE)

To define the sequences required for negative regulation, several WAP 5′ flanking subsequences were independently inserted into the multiple cloning site of the plasmid pT109luc [Nordeen, 1988]. This plasmid contains a firefly luciferase gene, the expression of which is driven by a herpes simplex virus thymidine kinase (tk) promoter (Fig. 2A). Luciferase activity was determined for each construct after transfection into CK cells and is presented relative to the activity obtained from transfection of the parental pT109luc plasmid (100%). Insertion of the EcoRI/SacI fragment of the WAP promoter (−2,400 to −1,400) did not affect expression from the tk promoter (Fig. 2A), and introduction of the SacI/XhoI fragment (−1,400 to −413) into pT109luc even resulted in a slight increase in luciferase activity (151%) compared to pT109luc (Fig. 2A). Thus neither of these fragments appears to carry a complete functional negative regulatory element (NRE). However, when the XhoI/KpnI fragment of the WAP promoter (−413 to +26) was inserted into pT109luc in the sense orientation, the expression of luciferase was dramatically reduced to about 10% of that obtained with pT109luc (Fig. 2A, pT.Xho/Kpn). Thus an NRE appears to be contained within this region that mediates a negative regulatory effect upon two heterologous promoters (hGH and tk).

To further define the sequences involved, subfragments of the XhoI/KpnI region were tested in the luciferase assay system. Insertion of the XhoI/XbaI subfragment (−413 to −93) decreased the expression of luciferase to similar levels as the XhoI/KpnI fragment (Fig. 2A, pT.Xho/Xba). Insertion of either of two subfragments of this minimal region, the XhoI/EcoNI fragment (−413 to −194) or the EcoNI/XbaI fragment (−194 to −93), resulted in only a slightly decreased (69%, pT.Xho/EcoN) or even a slightly increased (160%, pT.EcoN/Xba) level of luciferase expression. Essentially the same results were obtained upon transfection of the WAP-luciferase plasmids into XC and NIH3T3 cells (data not shown). These experiments define a negative regulatory element (NRE) as being located between the XbaI and XhoI sites at −93 and −413, respectively.

Orientation Dependence of the Negative Regulatory Element

In order to determine whether the WAP-NRE functions in an orientation dependent manner, the effect of the same WAP promoter fragments containing the NRE sequences inserted into the plasmid pT109luc in an antisense orientation was determined. The luciferase reporter plasmids pT.Xho/Kpn(as) and pT.Xho/Xba(as), carrying the WAP promoter fragments in the antisense orientation, were transiently transfected

into CK cells and the luciferase activity determined. Both WAP promoter fragments that conferred a negative regulatory effect upon the tk promoter in the sense orientation (Fig. 2B, pT.XhoI/KpnI and pT.XhoI/XbaI; open bars) showed only a very weak negative regulatory effect when inserted in the antisense orientation (Fig. 2B, 85%, pT.Xho/Kpn and 70%, pT.Xho/Xba; shaded bars). These results suggest that the WAP-NRE acts in an orientation dependent manner and thus resembles a transcription repressor [Tripputi et al., 1988; Vacher and Tilghman, 1990] rather than a silencer [Winoto and Baltimore, 1989].

Competition Assay With WAP Promoter Subfragments

To verify that the WAP promoter contains an NRE, and to confirm its location within the XhoI-XbaI fragment, competition assays were performed in which the WAP2-hGH plasmid was cotransfected with an eightfold molar excess of different subfragments of the WAP promoter (Fig. 3). Cotransfection of an excess of plasmid containing the entire 2.4 kb WAP promoter fragment (Poly3-WAP) with the WAP2-hGH construct resulted in a twentyfold increase in hGH expression (Fig. 3), compared to transfection of an excess of pUC19 with WAP2-hGH. The increase in hGH expression is presumably due the promoter region in Poly3-WAP competing for negative regulatory factors that would normally bind and downregulate WAP2-hGH expression and demonstrates that the concentration of these factors is limiting.

A number of promoter subfragments were assayed for their ability to compete for binding of putative negative regulatory factors and thus increase the expression of the WAP2-hGH construct. Cotransfection of a plasmid containing either the EcoRI/SacI fragment (−2,400 to −1,400) or the SacI/SacI fragment of the promoter (−1,400 to −358) had no appreciable effect upon WAP2-hGH expression (Fig. 3). In contrast, introduction of a plasmid containing the XhoI/XbaI fragment (−413 to −93) caused a 7.4-fold increase in expression of hGH from WAP2-hGH, suggesting that this 319 bp fragment contains at least a part of the NRE. The ability of subfragments of this XhoI/XbaI fragment to compete in this assay was also tested. In accordance with the luciferase data (Fig. 2),

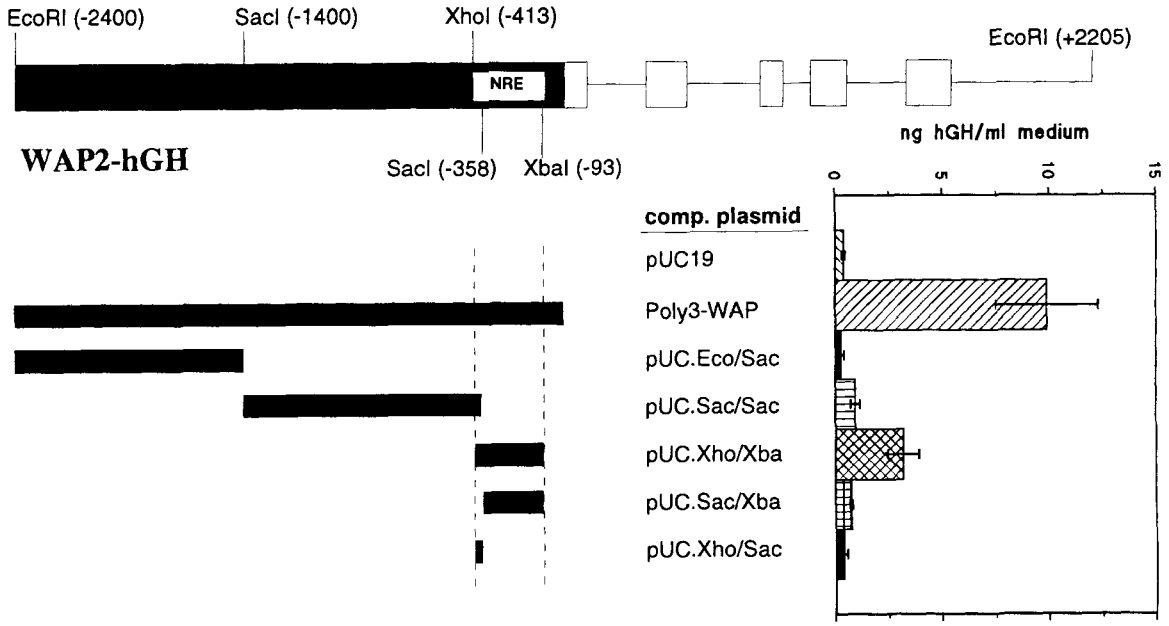


Fig. 3. WAP promoter fragments alleviate promoter repression. Schematic representation of the WAP competitor plasmids used in the assay. The length and location of WAP promoter sequences used as competitor are marked as black boxes. The exons of the hGH coding sequences are marked as open boxes. Each of the competitor plasmids, containing a WAP promoter fragment (black boxes) cloned into pUC19, was cotransfected in an eightfold molar excess with the reporter gene plasmid WAP2-hGH. Additionally the plasmid pT109luc was cotransfected as an internal control for transfection efficiency. A cotransfection of an eightfold molar excess of pUC19 (without any WAP promoter fragment) and WAP2-hGH was used as a negative control (pUC19). hGH protein expression after transient transfection was determined in five independent experiments and is presented as ng hGH/ml medium.

neither an XhoI/SacI (−413 to −358) nor a SacI/XbaI (−358 to −93) subfragment was able to compete in this assay. This suggests that the 5′ part of the XhoI/XbaI fragment is required for the negative regulatory activity. The 3′ part of the XhoI/XbaI fragment is also required as shown by the luciferase data (Fig. 2) in which XhoI/EcoNI and EcoNI/XbaI subfragments of the XhoI/XbaI fragment were also unable to confer a negative regulatory effect upon the tk promoter. Together the data from these two experiments suggest that the entire contiguous XhoI/XbaI fragment is required for efficient downregulation.

Gel Retardation Analysis of NRE-Protein Interactions

In order to determine whether potential negative regulatory proteins bind to the XhoI/XbaI region of the WAP promoter, a series of gel retardation experiments was performed. Nuclear extracts from CK cells were incubated with the XhoI/SacI (−413 to −358), SacI/XbaI (−358 to −93), and XhoI/XhoI (−413 to −93) fragments (Fig. 3). To exclude the possibility that the size of the DNA fragment affected the binding of nuclear proteins, vector sequences were included so that the DNA fragments used in the gel shift assay were of similar size (295–422 bp in length). A specific binding of CK nuclear proteins (Fig. 4A, Shift 1) to the entire XhoI/XbaI region was detected at low protein concentrations (lanes 13,14). Under these experimental conditions no specific binding of nuclear proteins prepared from CK cells either to the XhoI/SacI (lanes 1–5) or to the SacI/XbaI (lanes 6–10) region of the WAP promoter could be detected. These results are consistent with the entire XhoI/XbaI fragment being required for binding the nuclear (putative negative regulatory) factor NBF (NRE binding factor).

The ability of nuclear extracts from other, non-WAP expressing cell types to bind the XhoI/XbaI region was also investigated. Crude nuclear extracts isolated from NIH3T3 mouse fibroblasts (Fig. 4B, lane 8), XC rat fibrosarcoma cells (lane 10), and EF43 mouse mammary epithelial cells (lane 4) and incubated with the radiolabeled fragment showed the characteristic NBF activity in the gel shift assay. The specificity of this interaction was confirmed by the loss of the shift upon addition of an excess of nonlabeled XhoI/XbaI fragment as a specific competitor (Fig. 4B, lanes 3,5,9,11). In contrast, the addition of an excess of unlabeled XhoI/SacI or SacI/XbaI fragment did not abrogate the binding (data not shown). Although EF43 cells do not express WAP, they are mammary derived epithelial cells and may represent an early progenitor cell type [Günzburg et al., 1988, 1989]. Extracts were prepared from EF43 and XC cells after treatment with dexamethasone, insulin, and prolactin (Fig. 4B, lanes 6,7 and 12,13, respectively) to determine whether the binding of NBF to the NRE is influenced by pregnancy hormones. Specific binding to the XhoI/XbaI WAP promoter fragment was not altered by pretreatment of either cell line (lanes 6,12). Since nuclear proteins from all four cell lines retarded the DNA probe to the same extent, it is likely that the same protein or protein complex is involved. Similar results were also obtained with nuclear extracts from GR (mouse mammary adenocarcinoma) and Rat-2 (rat fibroblast) cells and from mouse liver (not shown). These results suggest that a nuclear protein which is a candidate for the putative negative regulatory factor (NBF) is present in many different cell types and is conserved between different mammalian species, such as mouse, rat, and cat.

Cell Type and Organ Specificity of NRE-NBF Interactions

The WAP promoter contains an NRE, deletion of which relieves promoter repression in vitro upon transfection of cells that do not express WAP. Gel shift experiments reveal that the NRE interacts with a binding factor NBF that appears to be present in all the non-WAP expressing cell lines tested. The complete 2.4 kb WAP promoter is not normally active in vitro [Eisenstein and Rosen, 1988; Chen and Bissell, 1989] or organs/tissues of the mouse other than the pregnant and lactating mammary gland [Piletz et al., 1981; Dandekar et al., 1982; Hennighausen et al., 1982; Hobbs et al., 1982], suggesting that the NRE-NBF interaction may play a role in determining tissue specificity. Gel shift assays were therefore performed to determine whether the NBF binding activity is present in extracts from lactating mammary glands which are permissive for WAP expression. As shown previously, nuclear extracts from CK cells gave the characteristic NRE-NBF band shift (Fig. 5A, lane 3 [NBF]) when incubated with the XhoI/XbaI fragment. In contrast, nuclear extracts derived from lactating mammary glands, upon incubation with the XhoI/XbaI fragment, yielded a novel band shift (Fig. 5A, lanes 4,5; Fig. 5B,

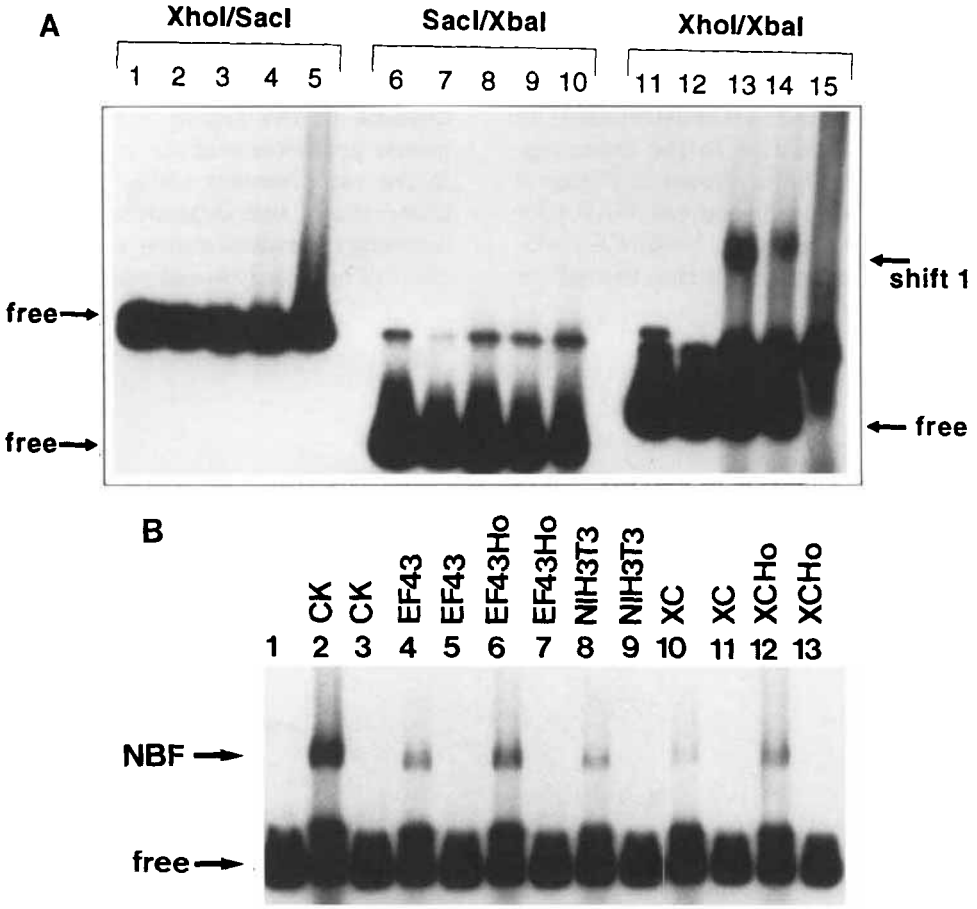


Fig. 4. A ubiquitous nuclear protein, NBF, binds to the WAP NRE. **A:** In the presence of 5 μg nonspecific competitor DNA (ds poly dIdC), 5 μg **(lanes 2,7,12)**, 10 μg **(lanes 3,8,13)**, 20 μg **(lanes 4,9,14)**, or 40 μg **(lanes 5,10,15)** of CK nuclear extracts were incubated with 1 ng of the indicated γ-^{32}P endlabeled XhoI/SacI (−413 to −358, lanes 1–5), SacI/XbaI (−358 to −93, lanes 6–10), and XhoI/XbaI (−413 to −93, lanes 11–15) fragments of the WAP promoter (Fig. 1A). To exclude the possibility that the size of the DNA fragment affected the binding of nuclear proteins, vector sequences were included so that the DNA fragments used in the gel shift assay were of similar size (XhoI/SacI: 422 bp; SacI/XbaI: 295 bp; XhoI/XbaI: 374 bp). **Lanes 1,6,11:** Control reactions without CK nuclear extracts. The positions of the free DNA and the DNA-NBF (shift 1) protein complex are indicated. **B:** In the presence of 5 μg poly dIdC, 10 μg of nuclear extracts from CK (feline kidney, **lanes 2,3**), EF43 (mouse mammary progenitor, **lanes 4–7**), NIH3T3 (mouse fibroblast, **lanes 8,9**), and XC (rat fibrosarcoma, **lanes 10–13**) cells was incubated with 1 ng of γ-^{32}P endlabeled XhoI/XbaI fragment. **Lane 1:** A control reaction without nuclear proteins. In lanes 3,5,7,9,11,13, 50 ng of cold specific competitor XhoI/XbaI fragment DNA was added to the binding reactions. Lanes 6,7,12,13 contain binding reactions with nuclear proteins isolated from EF43 and XC cells after induction of these cells with lactogenic hormones (10^{-6} M dexamethasone, 0.05U/ml insulin, and 0.01U/ml prolactin) 92 h prior to harvesting (indicated by Ho). The positions of free DNA and NBF retarded DNA-protein complex are indicated.

lanes 6,7, Shift 2) which was retarded considerably further than the NBF shift. This suggests that the protein(s) in the lactating mammary gland extracts that bind to the XhoI/XbaI fragment is distinct from or a modified form of those detected in cell extracts from CK and other non-WAP expressing cells. Further, lactating mammary gland extracts contain protein(s) that is able to interact with the XhoI/SacI subfragment of the NRE (Fig. 5B, lanes 2,3, Shift 3). This contrasts with the inability of the NBF factor present in CK nuclear proteins to bind to this fragment (Fig. 4A). Taken together the data suggest that two different factors bind to the NRE WAP promoter fragment; one is present in non-WAP expressing cells and requires the whole NRE fragment for binding, whereas the second factor, present in WAP expressing lactating mammary gland cells, binds to a subfragment of the functionally defined NRE.

Sequence Analysis of the NRE Region and Mapping of NBF Contact Points by Methylation Interference

The analysis of the exact location of the NRE binding site involved in the NRE-NBF interac-

tion required the determination of the mouse WAP promoter sequence since this had not been previously reported. Consequently, the sequence of the murine WAP 5′ region was determined up to nucleotide −620 (relative to the transcriptional initiation site) and is shown in Figure 6 alongside that of the published rat WAP promoter sequence [Campbell et al., 1984]. Comparison of the two sequences reveals that the mouse and rat sequences are highly homologous (82%) up to nucleotide −348. This is followed by a domain of little similarity between the sequences in the region −348 to −423 of the mouse promoter and the region −350 to −582 in the rat promoter (36%). Sequences further upstream of this domain again display a 81% homology between mouse and rat. The two regions of homology are as similar (82 and 81%) as

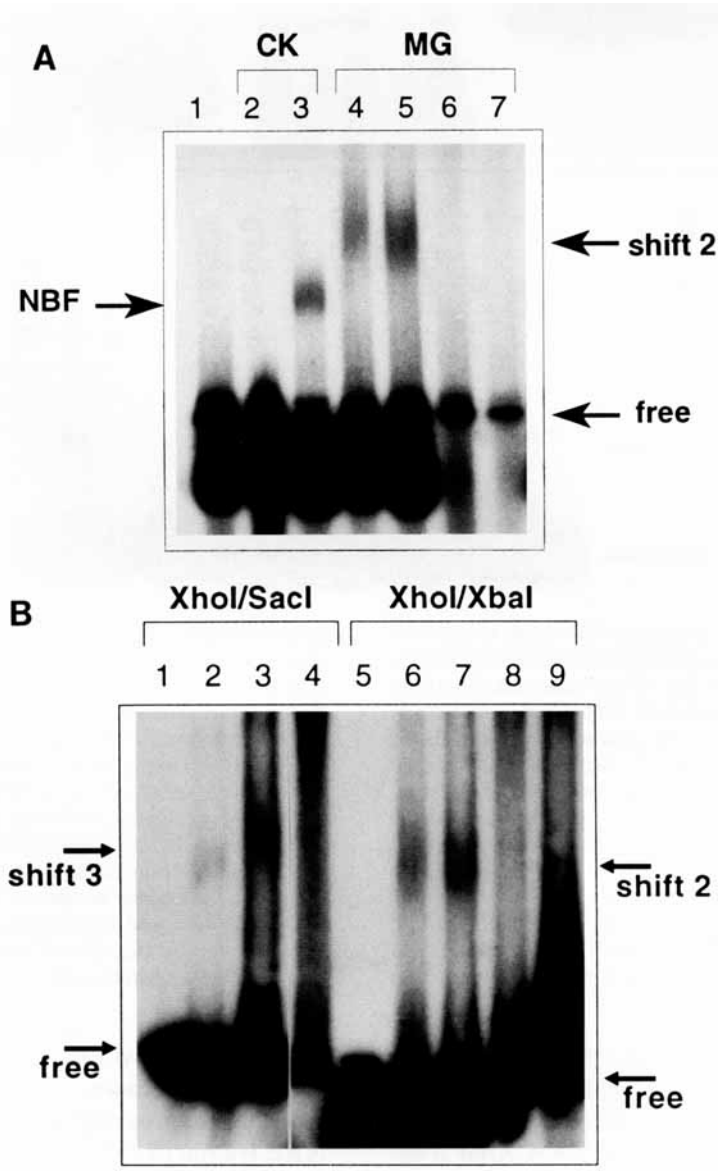


Fig. 5. A binding factor with different properties to NBF is present in lactating mammary gland extracts. **A:** In the presence of 5 μg poly dIdC, 5 μg **(lanes 2,4)**, 10 μg **(lanes 3,5)**, 20 μg **(lane 6)**, and 40 μg **(lane 7)** of nuclear extract from CK cells **(lanes 2,3)** or from lactating mammary gland (MG) **(lanes 4–7)** was incubated with 1 ng of γ-^{32}P endlabeled XhoI/XbaI fragment. **Lane 1:** A control reaction without any nuclear extract. The DNA-protein complex formed with CK cell extract is indicated NBF, whereas the lactating mammary gland extract gives a complex with a different mobility, indicated as shift 2. Supershifting was observed with 20 μg (lane 6) or 40 μg (lane 7) of lactating mammary gland extract. **B:** In the presence of 5 μg poly dIdC, 5 μg **(lanes 2,6)**, 10 μg **(lanes 3,7)**, 20 μg **(lanes 4,8)**, and 40 μg **(lane 9)** of nuclear extracts from lactating mammary gland was incubated with 1 ng of γ-^{32}P endlabeled XhoI/SacI (lanes 1–4) or XhoI/XbaI (lanes 5–9) fragment. Supershifting was again observed with 20 μg (lanes 4,8) or 40 μg (lane 9) of extract. **Lanes 1,5:** Control reactions without any nuclear proteins. The retarded DNA-protein complexes formed with the XhoI/XbaI and XhoI/SacI fragments are indicated shift 2 and shift 3, respectively.

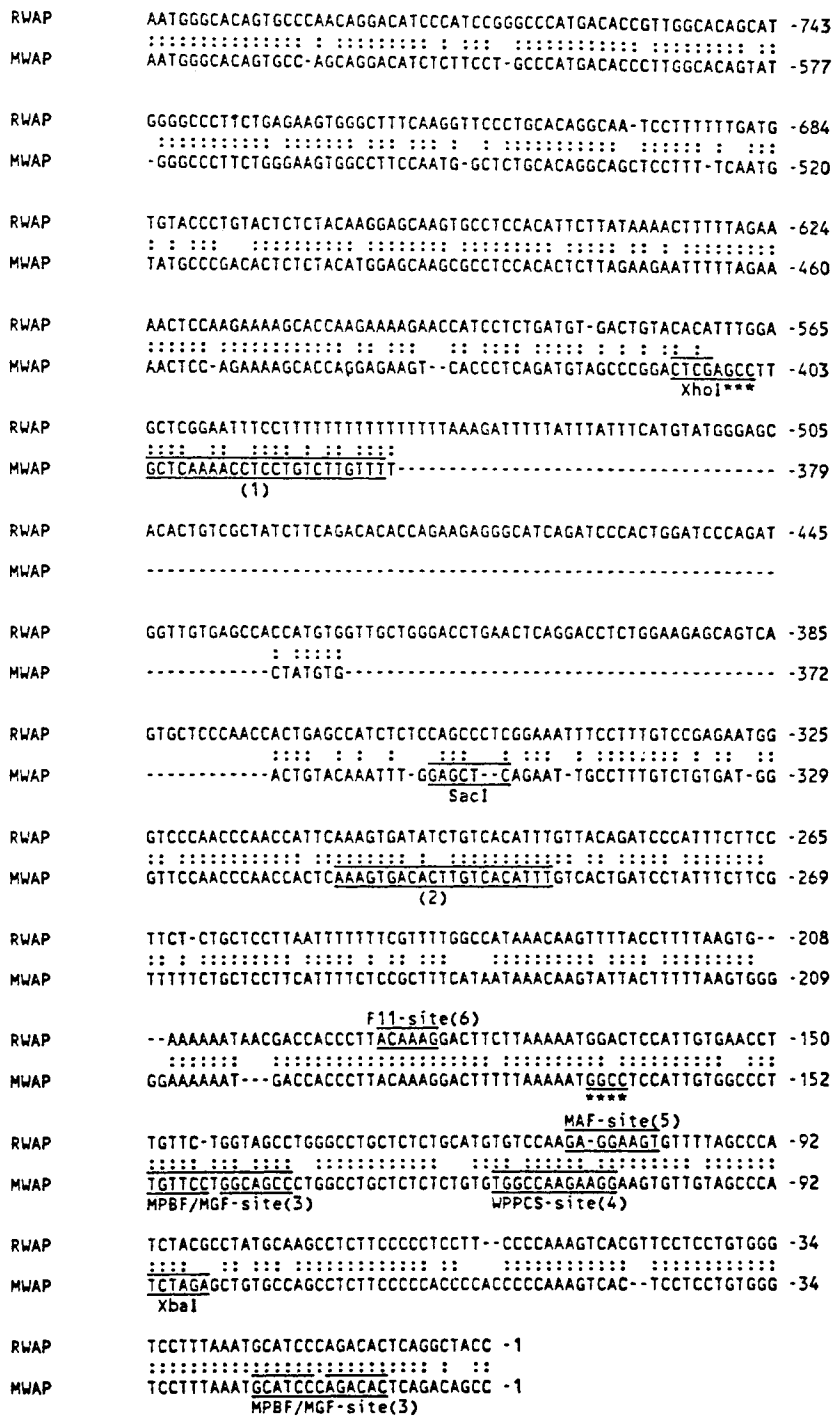


Fig. 6. Comparison of the 5' flanking sequences of the rat and mouse WAP genes. The mouse WAP promoter region was sequenced using a 23 nt primer with the sequence 5'AAACAAGACAGGAGGTTTTGAGCA3' complementary to the region −380 to −402 [underlined and indicated by (1)] and a 20 nt primer (5'AATGTGACAAGTGTCACTTT3') complementary to the region −292 to −311 [underlined and indicated by (2)]. The sequence of the promoter region was determined to nucleotide −638 and has been designated the GenBank accession number L21193. The mouse and rat [Campbell et al., 1984] WAP sequences were aligned using PC-Gene Software (IntelliGenetics, Mountain View, CA) with an open gap penalty of 10, extended gap penalty of 10, and a mismatch penalty of 3. Two binding sequences for the 89 kDa protein [Wakao et al., 1992] variously called milk protein binding factor [Watson et al., 1991] or mammary gland specific transcription factor [Schmitt-Ney et al., 1991, 1992] are indicated by (3). Also indicated is a sequence [whey protein promoter consensus sequence (4)] homologous to a binding site for a nuclear factor present in the rat alpha-lactalbumin gene [Lubon and Hennighausen, 1988]. The binding site for mammary cell activating factor [Mink et al., 1992] is indicated by (5), while (6) indicates the F11 factor binding site [Mink et al., 1992]. The binding site for the CK/liver nuclear protein (NBF) determined in this study is indicated by ***. Positions of relevant restriction sites are also indicated.

the sequences of the two rodent WAP coding regions (82%) [Campbell et al., 1984]. The functional significance of the sequence divergence in the −350 to −423/582 region is not evident, but part of the divergent region overlaps with the 5′ end of the NRE defined above.

In order to determine the exact location of the binding site of the nuclear proteins interacting with the XhoI/XbaI WAP promoter fragment (or NRE) in non-WAP expressing tissues and cells, methylation interference experiments were carried out with DNA fragments encompassing this region. XhoI/XbaI fragment (15 ng) selectively γ-^{32}P labeled at either end was randomly methylated at guanine residues and used in a gel shift analysis. Both the DNA/protein complex (shift) and the free DNA probe were excised, cleaved with piperidine, which cuts at every methylated guanine, and separated on a 6% denaturing polyacrylamide gel. Comparison of the piperidine cleavage pattern of the free DNA probe (Fig. 7A, lane F) with that of the shifted DNA/protein complex from CK cells (lane CK) or from nonexpressing liver (lane L) labeled at the XbaI site revealed that no cleavage was detectable in the shifted DNA/protein complex at the position of two neighboring guanine residues, whereas these positions were cleaved in the free DNA (Fig. 7A, arrows). This suggested that these two methylated guanines were not represented in the shifted DNA/protein complex and thus that methylation of these two residues, located at positions −167 and −168 in the sequence −164 GGA**GG**CCA −171 (lower strand), probably precluded the formation of the DNA-protein complex with nuclear extracts from non-WAP expressing tissues and cells. No other major differences in the cleavage pattern of the free and the complexed DNA could be detected.

The complementary methylation interference experiment using an XhoI/XbaI WAP promoter fragment labeled on the complementary DNA strand at the XhoI end identified two GG residues at positions −169 and −170 (data not shown), directly next to, but on the complementary strand to, the two guanines (−167 and −168) mapped before as involved in DNA protein interaction. This suggests that the palindromic sequence GGCC is intimately involved in the binding of the protein(s) that generates the NBF-NRE gel shift.

Additionally this methylation interference experiment revealed that methylation of one guanine residue at the 5′ end of the probe interferes with the binding of CK nuclear proteins to the XhoI/XbaI fragment (Fig. 7B). One cleavage product, 21 bp from the labeled 5′ end (arrow), can be observed in the free DNA (lane F) but not in the complexed DNA (lane C), implying that methylation of this guanine residue also interferes with the binding of NBF to the NRE.

These data suggest that the nuclear protein(s) comprising NBF and binding to the NRE makes contact not only at position −167 to −170 but also at position −408 (Fig. 7). These findings are in accordance with the results obtained in the gel shift assay, where neither the XhoI/SacI fragment (containing the contact point −408) nor the SacI/XbaI fragment (containing the contact points −167 to −170) is sufficient alone to bind NBF, whereas the entire XhoI/XbaI fragment is (Fig. 4A).

Mutation Analysis of Contact Points

If the mapped contact points are critical for NBF binding to the NRE, mutation of these nucleotides in vitro should interfere with the binding of NBF. Further, if NBF is responsible for the suppression of transcription from the WAP promoter, such mutated NRE fragments should no longer be able to function in the competition assay. In order to test these hypotheses, point mutations were introduced at one or both of the two contact sites. The −170 GGCC −167 motif was converted to GAAT, and the −410 GAGCCTTG −403 sequence was converted to GACGTTTG (Fig. 8A). Figure 8B shows a gel shift analysis of the nonmutated XhoI/XbaI fragment (lane 2), compared to fragments carrying mutations at the 5′ contact point (NREm5′; lane 4), the 3′ contact point (NREm3′; lane 6), or in both contact points (NREm5′/3′; lane 8). Mutation of the 3′ contact point (−170 GGCC −167) severely reduces the ability of the NRE to bind NBF. In contrast, however, mutation of the 5′ contact point did not appear to affect the ability of the mutated NRE to bind NBF. This suggests that either the sequence −410 GAGCCTTG −403 is not involved inbinding NBF or that this contact point plays a secondary, possibly stabilizing role for NBF binding.

To investigate this further, the effect of the NRE mutants on transcription was analyzed in a competition assay. The NRE or NRE mutants were cotransfected in a 10:1 molar ratio with the plasmid pT.Xho/Xba which contains the functional WAP-NRE linked to the reporter geneplasmid pT109luc (Fig. 2). As expected, a tenfold excess of the nonmutated NRE fragment as

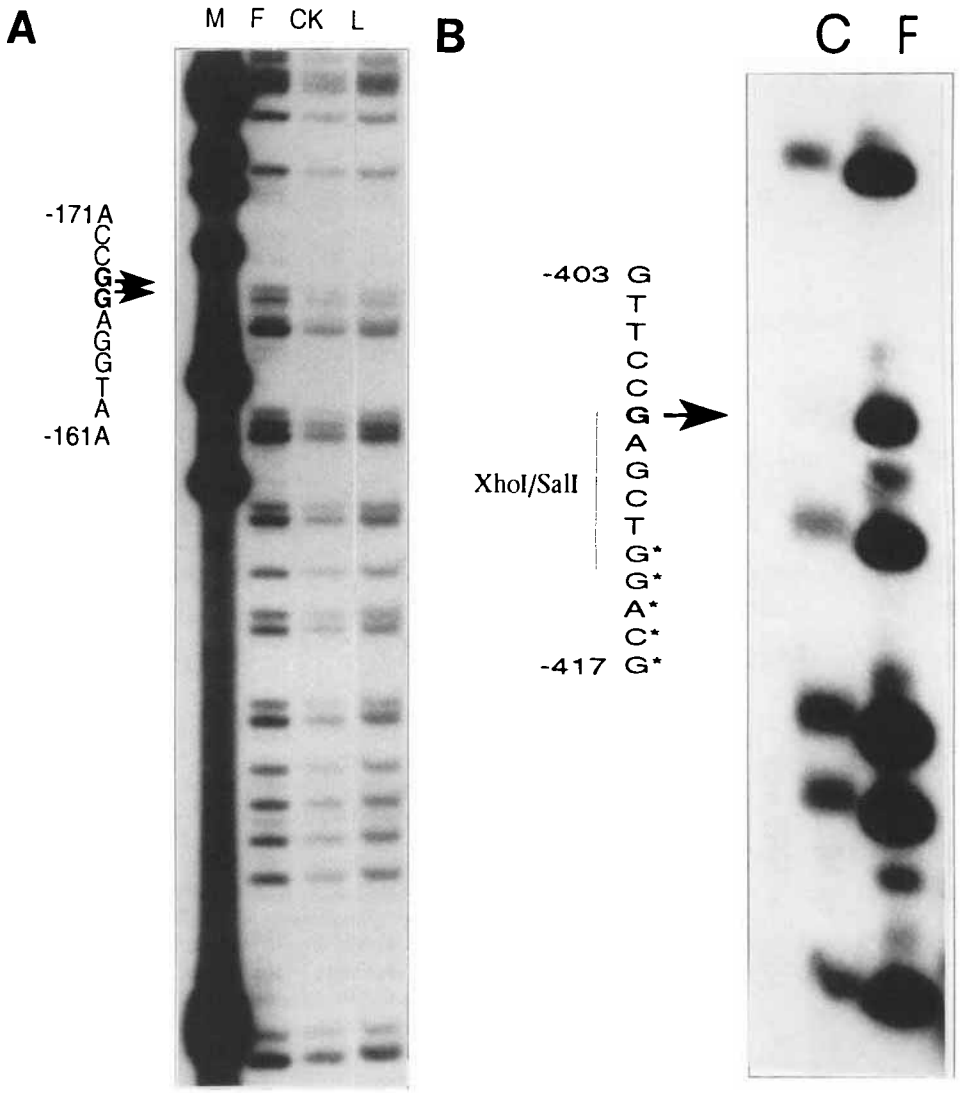
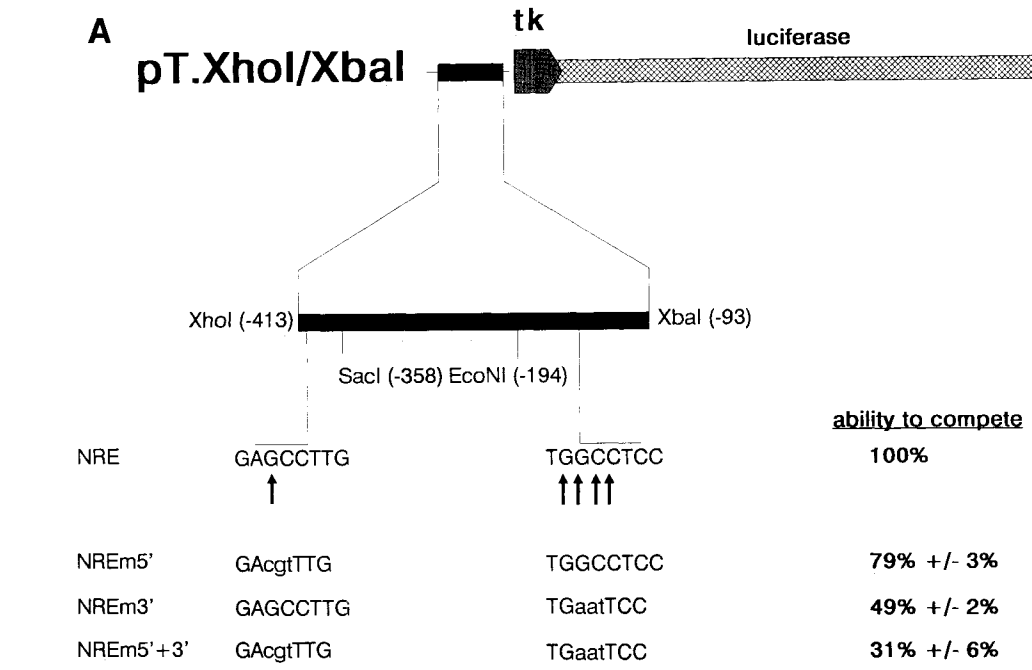


Fig. 7. Methylation interference assay of the WAP NRE. **A:** In the presence of 5 μg poly dIdC, 10 μg of nuclear extracts isolated from CK cells **(lane CK)** or from murine liver **(lane L)** was incubated with 1 ng of XhoI/XbaI fragment labeled with γ-^{32}P at the XhoI end and methylated by DMS treatment. The free and retarded DNA bands were excised from a subsequent gel retardation analysis, cleaved with piperidine, and separated on a sequencing gel. **Lane M:** A DNA size marker (pBR322 digested with MspI). **Lane F:** The piperidine cleavage products of uncomplexed XhoI fragment. Cleavage products absent from the complexed DNA are indicated by arrows. The DNA sequence around the uncleaved G residues and the localization with respect to the published start of the WAP coding sequence are indicated. **B:** In the presence of 5 μg poly dIdC, 10 μg of nuclear extract from CK cells **(lane C)** was incubated with 1 ng of XhoI/XbaI fragment labeled at the XbaI end with γ-^{32}P and methylated by DMS treatment. The free and retarded DNA bands were excised after gel retardation, cleaved with piperidine, and separated on a sequencing gel. **Lane F:** The piperidine cleavage products of uncomplexed endlabeled XhoI/XbaI fragment. The DNA sequence around the uncleaved G residues and the localization with respect to the published start of the WAP coding sequence are indicated. The XhoI/XbaI fragment used for methylation interference was cloned into the SalI and XbaI sites of pUC19, and these vector sequences are starred.

competitor relieves repression of expression from pT.Xho/Xba, leading to a tenfold increase in luciferase expression as compared to transfection of pT.Xho/Xba alone. This expression level is arbitrarily set as 100% (Fig. 8A). These data confirm the previous observation that an excess of free NRE can efficiently bind NBF and thereby relieve the NBF mediated repression of the test tk promoter. A tenfold molar excess of the 5′ contact point NRE mutant (NREm5′) also competed for binding of NBF reasonably well (Fig. 8A) (79%), and gel shift analysis confirms that the NREm5′ fragment is able to bind to NBF to a similar extent as nonmutated NRE (Fig. 8B, lane 4). In contrast, mutation of the 3′ contact point (NREm3′) diminished the ability of this



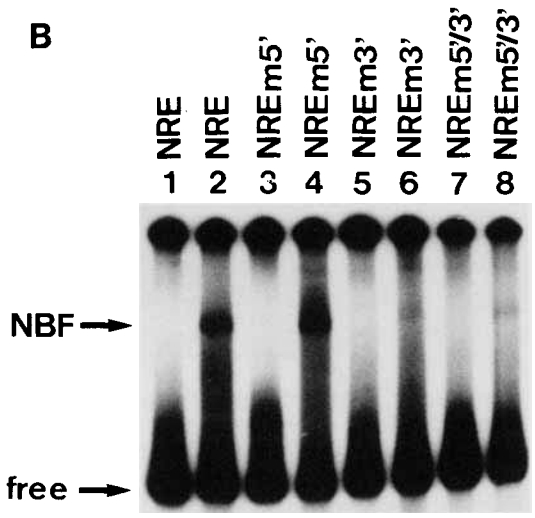


Fig. 8. Mutation analysis of the WAP-NRE. **A:** Schematic representation of the reporter gene plasmid pT.XhoI/XbaI. The WAP XhoI/XbaI fragment (black box) is shown enlarged, and the G residues contacted by NBF are arrowed. The mutated WAP-NRE fragments are indicated below, and mutated nucleotides are marked as lower case letters. The relative efficiency of the nonmutated and mutated NREs to compete with pT.Xho/Xba and thereby alleviate repression of luciferase expression is shown. **B:** NRE fragments containing the nonmutated and mutated bases were PCR amplified, γ-^{32}P endlabeled, and used in gel retardation assays. **Lanes 1,3,5,7:** Control reactions without nuclear extracts. **Lanes 2,4,6,8:** Reactions with CK nuclear extracts. Lanes 1,2 contain binding reactions of the nonmutated PCR product (NRE). Lanes 3,4 contain binding reactions with the PCR product NREm5' (carrying a mutation in the 5' binding site only). Lanes 5,6 contain binding reactions with NREm3' (carrying a mutated 3' binding site), whilst lanes 7,8 contain binding reactions with NREm5'/3' which is a PCR product carrying mutations at both binding sites. The positions of free and NBF complexed DNA are indicated.

fragment to compete for NBF in the luciferase expression assay (Fig. 8A) (49%) which is presumably due to a reduced ability to bind NBF in the gel shift assay (Fig. 8B, lane 6). An NRE carrying mutations at both sites (NREm5'/3') showed a severely reduced activity in the competition assay (Fig. 8A) (31%) and of all the fragments showed consistantly the poorest binding in gel shifts (Fig. 8B, lane 8). These results clearly show that both contact points are involved in NBF-NRE binding and that mutation of both virtually eliminates this binding and the associated transcriptional downregulation, but that the 3' contact point may be more important for this activity.

DISCUSSION

Whey acidic protein (WAP) is specifically expressed in the mammary gland during pregnancy and lactation. Lactogenic hormone specific activation of milk gene expression has recently been shown to be mediated by an 89 kDa protein [Wakao et al., 1992] variously termed mammary gland specific transcription factor (MGF) [Schmitt-Ney et al., 1991, 1992] or milk protein binding factor (MPBF) [Watson et

al., 1991]. Two putative MGF/MPBF binding sites are present in the WAP promoter (Fig. 6), implying that this factor may be involved in lactogenic hormone induced expression of WAP [Watson et al., 1991]. A second factor, MAF, also has been shown to activate the expression of mammary specific genes, including WAP [Mink et al., 1992]. MAF binds to the core sequence −110 GAAGGAAGT −102 but requires the binding of NFI to two sites flanking the MAF core motif, as well as an second factor, F11, which binds to an ACAAAG motif (Fig. 6). Thus, the induction of WAP expression during pregnancy is likely to be controlled by a number of orchestrated protein DNA interactions.

However, a number of cell types other than mammary epithelial cells respond to pregnancy hormones but do not express WAP. This suggests that additional regulatory mechanisms may exist to specifically suppress the expression of WAP in nonmammary cells, thereby also ensuring the tissue specific expression of this gene. Using both deletion and competition analyses we have demonstrated that the 5′ noncoding region of the WAP gene contains an element that represses transcription from linked heterologous promoters. This negative regulatory element (NRE) is functionally located between −93 and −413 (Fig. 2) upstream of the WAP transcriptional initiation site [Campbell et al., 1984]. Subfragments of this region were not able to function as an NRE in these experiments. Further, the WAP-NRE functions in an orientation dependent fashion (Fig. 2B), a property characteristic of a transcriptional repressor [Tripputi et al., 1988; Vacher and Tilghman, 1990].

The factors that interact with the NRE and therefore that potentially mediate the observed transcriptional repression were characterized by gel shift analyses. Initial experiments using nuclear extracts from CK and NIH3T3 cells (used to define the NREs in the deletion and competition analyses), revealed that these cells contain a nuclear protein or protein complex, termed NBF (NRE binding factor), that interacts with the complete NRE containing fragment (i.e., −93 to −413) of the WAP promoter (Fig. 4). Subfragments of this region were not shifted by NBF present in the cell extracts. In contrast, extracts isolated from lactating mammary gland did not show the characteristic NBF shift, but instead a novel shift was detected when the NRE containing XhoI/XbaI fragment was used. Further, this novel shift was detected using the XhoI/SacI subfragment and the lactating mammary gland extract (but not with the CK extract). This could mean either that NBF is not expressed in the epithelial cells isolated from lactating mammary gland or that it is present in a form that precludes its binding to the NRE. Thus NBF binding activity correlates with the inability to express WAP. This suggests that the NBF protein or protein complex that physically interacts with the WAP-NRE may be involved in mediating the negative regulation. Preliminary data indicate that a 53 kDa protein binds to the NRE. This protein is present in extracts from a number of cell lines that are unable to express WAP transgenes efficiently, including CK cells and mammary derived EF43 [Günzburg et al., 1988] and GR [Ringold et al., 1975] cells as well as from mouse organs such as liver that do not express WAP. However the 53 kDa protein is not detectable in extracts prepared from lactating mammary gland (unpublished data).

Methylation interference experiments enabled the contact points between NBF and NRE to be partially characterized (Fig. 7). Disruption of these contact points by site directed mutagenesis revealed that DNA fragments that show a diminished ability to bind NBF are no longer active in a functional competition assay (Fig. 8). This provides confirmatory evidence that the interaction between NRE and NBF is involved in mediating the negative regulatory effect.

Recently, the promoters of two other genes which are specifically expressed in the lactating mammary gland have been shown to contain negative regulatory elements in their promoters. One of these genes encodes the milk protein β-casein. In similar analyses to those used in this study of the WAP promoter, two nuclear factors were identified that bind to the β-casein promoter. Site directed mutagenesis studies showed that loss of binding of these factors resulted in an elevated basal activity from the β-casein promoter [Schmitt-Ney et al., 1991]. However, the recognition sequences for the β-casein and WAP transcriptional repressors are not homologous. The expression of mouse mammary tumor virus (MMTV), a retrovirus which is also preferentially expressed in lactating mammary glands, is also subject to negative regulation [reviewed in Günzburg and Salmons, 1992]. The long terminal repeat (LTR) of MMTV, which has recently been shown to contain two promoters [Günzburg et al., 1993], has been reported to carry numerous NREs [Morley et al., 1987; Mink

et al., 1990; Ross et al., 1990; Gouilleux et al., 1991], some of which act synergistically [Lee et al., 1991]. Interestingly, a 0.6 kb Pst I fragment from the MMTV LTR [Salmons et al., 1985] containing most of the previously described NREs can compete with the WAP-NRE for NBF binding (unpublished data). The presence of NREs in the promoter regions of a number of genes that are preferentially expressed in lactating mammary glands (WAP, β-casein, and MMTV) suggests that negative regulation may be a common mechanism for determining mammary specificity.

The transcription factor NF-1 has been implicated in transcriptional control of MMTV [Bruggemeier et al., 1990]. More recently, mammary specific forms of NF-1 could be shown to interact with β-lactoglobulin, the major whey protein in the milk of ruminants [Watson et al., 1991]. The NBF protein that binds to the NRE is not related to NF-1 or to other relatively well-characterized transcription factors such as AP-1 or SP-1 since oligonucleotides carrying the binding sites for each of these factors failed to compete with the NRE for binding to NBF (unpublished data).

Both the deletion analysis and the gel shift analysis revealed that a relatively large DNA fragment (319bp) mediates the negative regulatory effect (NRE) and is required to obtain the gel shift. Subfragments of this region no longer act as an NRE and are not shifted by NBF. It is most unusual that such a large DNA region acts as a response element [Pabo and Sauer, 1992]; however, it is conceivable that DNA secondary structure brings these two sites close together, allowing them to be contacted by the same factor or factor complex in a similar fashion as has been proposed for the interaction between distant enhancer elements and the TATA box [reviewed by Struhl, 1991]. The protein (complex) interacting with the WAP-NRE contacts the DNA at two sites (GAGCCT at −408; TGGCCT at −170 to −167) which, although not identical, share a GCCT core sequence (Fig. 7). Although the functional significance of two contact points is not yet clear, the −170 to −167 core sequence seems to contribute more to both NBF binding and transcriptional downregulation (Fig. 8).

The studies described in this paper provide evidence for a DNA element present in the WAP promoter and a factor which binds to this element, thereby repressing expression from linked promoters. The presence of the NRE in the WAP promoter suggests that it is responsible, at least in part, for the repression of endogenous WAP gene expression in nonmammary cells [Lubon and Hennighausen, 1987; Eisenstein and Rosen, 1988]. It seems likely that such negative control mechanisms complement the previously described positive regulatory mechanisms (MGF/MPBF, MAF, F11) to prevent inappropriate expression of WAP. Recently it has been shown that the WAP protein shares homology with protease inhibitors and that precocious expression of WAP results in a *milchlos* phenotype characterised by defects in mammary development and lactation [Burdon et al., 1991b; Shamay et al., 1992]. Clearly, the expression of a protein that has such far-reaching effects for tissue/organ architecture and development must be very tightly regulated.

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